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Cation-selectivity of the L-glutamate transporters of *Escherichia coli*, *Bacillus stearothermophilus* and *Bacillus caldotenax*: dependence on the environment in which the proteins are expressed

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Summary

L-Glutamate transport by the H^+–glutamate and Na^+–glutamate symport proteins of *Escherichia coli* K-12 ($GltP_{Ec}$ and $GltS_{Ec}$, respectively) and the Na^+–H^+–glutamate symport proteins of *Bacillus stearothermophilus* ($GltT_{Bs}$) and *Bacillus caldotenax* ($GltT_{Bc}$) was studied in membrane vesicles derived from cells in which the proteins were either homologously or heterologously expressed. Substrate and inhibitor specificity studies indicate that $GltP_{Ec}$, $GltT_{Bs}$ and $GltT_{Bc}$ fall into the same group of transporters, whereas $GltS_{Ec}$ is distinctly different from the others. Also, the cation specificity of $GltS_{Ec}$ is different; $GltS_{Ec}$ transported L-glutamate with (at least) two Na^+, whereas $GltP_{Ec}$, $GltT_{Bs}$ and $GltT_{Bc}$ catalysed an electrogenic symport of L-glutamate with $\geq$ two H^+, i.e. when the proteins were expressed in *E. coli*. Surprisingly studies in membrane vesicles of *B. stearothermophilus* and *B. caldotenax* indicated a Na^+–H^+–L-glutamate symport for both $GltT_{Bs}$ and $GltT_{Bc}$. The Na^+ dependency of the GltT transporters in the *Bacillus* strains increased with temperature. These observations suggest that the conformation of the transport proteins in the *E. coli* and the *Bacillus* membranes differs, which influences the coupling ion selectivity.

Introduction

In the mesophile *Escherichia coli* three L-glutamate transport systems have been identified: (i) a binding-protein-dependent sodium-independent system; (ii) a binding-protein-independent sodium-independent system ($GltP_{Ec}$); and (iii) a binding-protein-independent sodium-dependent system ($GltS_{Ec}$) (Halpern *et al.*, 1973; Miner and Frank, 1974; Schellenberg and Furlong, 1977). In the thermophilic bacteria *Bacillus stearothermophilus* and *Bacillus caldotenax*, only one L-glutamate transport system is present: $GltT_{Bs}$ and $GltT_{Bc}$, respectively (Tolner *et al.*, 1992a). The transport by the GltT proteins is driven by the proton motive force (Δp) and also by inwardly directed Na^+ gradients (ΔpNa), and transport of L-glutamate probably occurs in symport with one H^+ and one Na^+ (De Vrij *et al.*, 1989; Heyne *et al.*, 1991). A similar type of Na^+–H^+–glutamate symport has been found in the thermophile *Bacillus* IS1 ($GltT_{Bi}$) (B. Tolner and B. Poolman, unpublished). In order to discriminate between Na^+–glutamate, H^+–glutamate and Na^+–H^+–glutamate transport proteins, the gene designation *gltS*, *gltP* and *gltT* is used. Additionally, the subscripts Bs, Bc, Bi, Bsu and Ec are used to discriminate between the genes/proteins of *B. stearothermophilus*, *B. caldotenax*, *B.* IS1, *B. subtilis* and *E. coli*, respectively. The glutamate transporters $GltS_{Ec}$, $GltP_{Ec}$ and $GltT_{Bs/Bc/Bi}$ seem to differ from each other with respect to their cation selectivity. So far, the kinetic parameters of transport and the cation and substrate specificity of $GltP_{Ec}$ and $GltS_{Ec}$ have only been determined in membrane vesicles and intact cells in which multiple glutamate transport systems were expressed, and under conditions in which ion gradients and Na^+ concentrations were not controlled. In order to enable a better comparison of the different transport proteins, the energetic and cation coupling properties and the substrate specificity of $GltP_{Ec}$ (*E. coli* K-12), $GltS_{Ec}$ (*E. coli* K-12), $GltT_{Bs}$ and $GltT_{Bc}$ were studied after expression in a genetically well-defined *E. coli* strain (ECOMUT1). Transport studies in membrane vesicles of this *E. coli* ECOMUT1 indicate that L-glutamate transport by $GltS_{Ec}$ occurs by electrogenic sodium symport, whereas $GltP_{Ec}$, $GltT_{Bs}$ and $GltT_{Bc}$ transport L-glutamate by electrogenic proton symport. $GltT_{Bs}$- and $GltT_{Bc}$-mediated transport is dependent on Na^+ and exhibits a 10-fold decreased affinity constant for L-glutamate uptake when the proteins are expressed in their original lipid environment.

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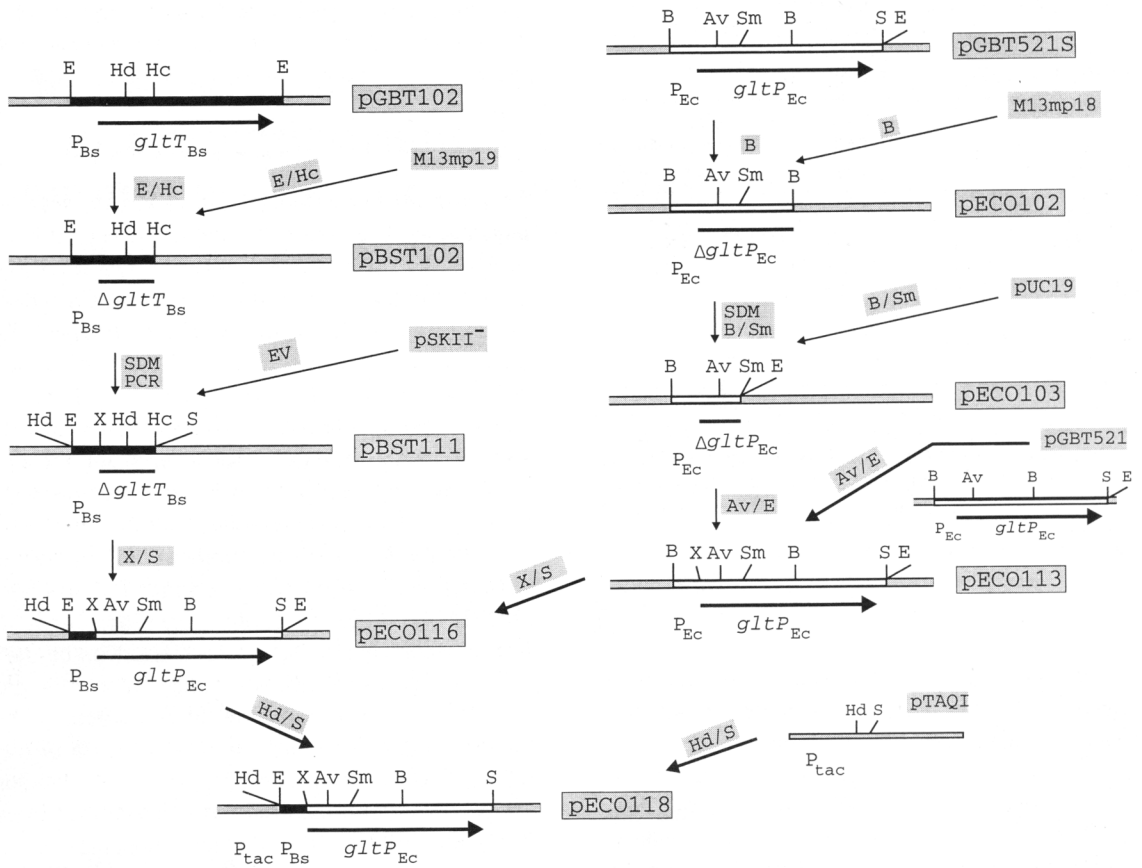

Fig. 1. Construction of plasmid pECO118. (i) The 546 bp *Eco*RI–*Hin*cII fragment (*gltT* promoter/RBS region) of pGBT102 was ligated into phage M13mp19 (pBST102) and a *Xba*I site (primer: 5′-AAAAGAAAGGGGCTCTAGAATGAGAAAAATTGG-3′) was introduced immediately upstream of the translation initiation codon of *gltT* (pBST100). Subsequently, the *gltT* promoter/RBS region of pBST100 was isolated as a PCR product (primers: T7 universal and M13 reverse) and ligated into *Eco*RV-digested pSKII⁻ to form pBST111. (ii) The 941 bp *Bam*HI fragment (*gltP* promoter/RBS region) of pGBT521 was ligated into phage M13mp18 (pECO521) and a *Xba*I site (primer: 5′- CCATTGAGGAAGTCTCTA-GAATGAAAAATATAAAATTCAGCC-3′) was introduced immediately upstream of the translation initiation codon of *gltP* (pECO100). The *Sma*I–*Bam*HI *gltP* promoter/RBS region of pECO100 was ligated into *Sma*I/*Bam*HI-digested pUC19 (pECO103) and, subsequently, the *Ava*I–*Eco*RI *gltP* fragment of pGBT521 was ligated into *Ava*I/*Eco*RI-digested pECO103, yielding pECO113. By substituting the 121 bp *Xba*I–*Sac*I fragment of pBST111 for the *Xba*I–*Sac*I fragment (*gltP*-coding region) of pECO113, the *gltP* gene was placed behind the *gltT* promoter/RBS region (pECO116). The *Hin*dIII–*Sac*I fragment was ligated into *Hin*dIII/*Sac*I-digested pTAQI to form pECO118, which has P_{tac} and P_{bs} in tandem 5′ of the *gltP* gene.

Symbols: Av, B, E, Ev, Hc, Hd, S, Sm and X represent *Ava*I, *Bam*HI, *Eco*RI, *Eco*RV, *Hin*cII, *Hin*dIII, *Sac*I, *Sma*I and *Xba*I restriction endonuclease, respectively. P_{Bs}, P_{Ec} and P_{tac} correspond to the *gltT*, and *gltP* promoter/RBS region, and *tac* promoter, respectively. Black, open and shaded boxes correspond to *gltT* fragment, *gltP* fragment and vector sequence, respectively. Arrow below sequence: coding region of respective gene. SDM, site-directed mutagenesis.

Results

Expression of L-glutamate transport proteins

In membrane vesicles derived from ECOMUT1/pKK223-3, L-glutamate transport was completely absent, as expected since both $gltS_{Ec}$ and $gltP_{Ec}$ were inactivated in this strain. Membrane vesicles of ECOMUT1/pGBT521 ($GltP_{Ec}$), in which $gltP_{Ec}$ was expressed from its own promoter/RBS (ribosome-binding site), exhibited in the presence of potassium ascorbate (K-asc)/phenazine methosulphate (PMS) an initial L-glutamate uptake rate of 0.03 nmol min mg^{-1} protein. A much higher initial rate of uptake (0.56 nmol min mg^{-1} of protein) was observed when the coding region of $gltP_{Ec}$ was fused to the promoter/RBS region of $gltT_{Bs}$ (Fig. 1, for details on the construction of pECO118). $GltT_{Bs}$- and $GltT_{Bc}$-mediated transport in membrane vesicles of ECOMUT1(pGBT112) ($GltT_{Bs}$) and ECOMUT1(pGBT231) ($GltT_{Bc}$), respectively, occurred with initial uptake rates of 0.78 and 0.80 nmol min mg^{-1} protein, respectively. Using membrane vesicles of ECOMUT1(pMK15) ($GltS_{Ec}$), the initial rate of $GltS_{Ec}$-mediated transport was 1.98 nmol min mg^{-1} protein. The transport rates were measured in duplicate (less than 10% variation between the two measurements) and the assays were performed in buffer containing 50 mM NaCl. Without added NaCl, the buffer contained <10 μM Na^+ but the transport rates for $GltP_{Ec}$, $GltT_{Bs}$ and

Table 1. Kinetic parameters of glutamate transport[a] in membrane vesicles of *B. stearothermophilus* ($GltT_{Bs}$), *B. caldotenax* ($GltT_{Bc}$), and *E. coli* ECOMUT1 harbouring pECO118 ($GltP_{Ec}$), pMK15 ($GltS_{Ec}$), pGBT112 ($GltT_{Bs}$) or pGBT231 ($GltT_{Bc}$).

Vesicles derived from	K_m^{app} (μM)	V_{max}^{app} (nmol min^{-1} mg protein^{-1})
ECOMUT1/pECO118 ($GltP_{Ec}$)	3.6 ± 1.2	6.2 ± 0.4
ECOMUT1/pMK15 ($GltS_{Ec}$)	31.6 ± 11.1	37.7 ± 3.1
ECOMUT1/pGBT112 ($GltT_{Bs}$)	56.9 ± 18.3	24.2 ± 2.3
ECOMUT1/pGBT231 ($GltT_{Bc}$)	38.0 ± 13.6	16.8 ± 1.3
B. stearothermophilus ($GltT_{Bs}$)	5[b]	11[b]
B. caldotenax ($GltT_{Bc}$)	3[c]	17[c]

a. Uptake experiments were performed by diluting membrane vesicles 100-fold into oxygen-saturated 50 mM potassium phosphate, pH 6.0, 5 mM $MgSO_4$, 50 mM NaCl, 10 mM K-asc and 100 μM PMS. After 1 min of incubation, L-[^{14}C]-glutamate (2–400 μM) was added and transport assays were further handled as described in the *Experimental procedures.*
b. Data taken from Heyne *et al.* (1991).
c. Data taken from Tolner *et al.* (1992a).

$GltT_{Bc}$ were essentially the same; L-glutamate uptake by GltS, however, was only detectable in the presence of added NaCl (see also below).

Kinetics of L-glutamate uptake

The kinetics of L-glutamate uptake mediated by the different glutamate transporters in membrane vesicles of *E. coli* ECOMUT1 were determined in the presence of 50 mM NaCl. The apparent affinity constants (K_m^{app}) for L-glutamate uptake via $GltS_{Ec}$, $GltT_{Bs}$ and $GltT_{Bc}$ were in the range of 30–60 μM. A significantly lower K_m^{app} was observed for $GltP_{Ec}$ (Table 1). Interestingly, the K_m^{app} values for L-glutamate uptake by $GltT_{Bs}$ and $GltT_{Bc}$ in membrane vesicles of the parent *Bacillus* strains are in the range of 3–5 μM, which is significantly lower than when the proteins are expressed in *E. coli.*

Substrate specificity

The solutes used to examine the substrate specificity of the L-glutamate transporters $GltP_{Ec}$, $GltS_{Ec}$, $GltT_{Bs}$ and $GltT_{Bc}$ are listed in Table 2. The apparent inhibition constants of L-glutamate transport (K_i^{app}) were determined from the initial rate of L-glutamate uptake in the absence and presence of excess unlabelled substrate, using the K_m^{app} and V_{max} values presented in Table 1. $GltP_{Ec}$, $GltT_{Bs}$ and $GltT_{Bc}$ were found to be highly specific for L-glutamate, L-aspartate, β-hydroxyaspartate (βHA) and cysteic acid. D-Glutamate was somewhat inhibitory whereas α-methyl-glutamate (αMG), L-glutamine and L-asparagine had no significant effect on the transport rate, not even at a 500-fold excess of the substrates (K_i^{app} > 1 mM; Table 2). By comparison, $GltS_{Ec}$ showed a high affinity for L-glutamate and low affinity for D-glutamate and αMG (Table 2).

Effect of ionophores on L-glutamate transport

To establish the nature of the cations transported with L-glutamate, the effect of ionophores on sodium and/or proton motive force-driven transport (at pH 6.0) was studied in membrane vesicles of *B. stearothermophilus*, *B. caldotenax* and *E. coli* ECOMUT1 harbouring pECO118 ($GltP_{Ec}$), pGBT112 ($GltT_{Bs}$), pGBT231 ($GltT_{Bc}$) or pMK15 ($GltS_{Ec}$).

Both nigericin (electroneutral exchange of K^+/H^+) as well as valinomycin (K^+ ionophore) inhibit the uptake of L-glutamate via $GltP_{Ec}$ partially, while complete inhibition was observed in the presence of both ionophores. Also, L-glutamate transport mediated by $GltT_{Bs}$ and $GltT_{Bc}$ were similarly affected by valinomycin and nigericin. Furthermore, the presence of an additional sodium motive force (50 mM NaCl in the assay buffer; the standard assay buffer contained <10 μM Na^+) had no effect on L-glutamate transport by $GltP_{Ec}$, $GltT_{Bs}$ and $GltT_{Bc}$. These observations strongly suggest that L-glutamate transport mediated by $GltP_{Ec}$, $GltT_{Bs}$ and $GltT_{Bc}$ in *E. coli* is an electrogenic process in which only protons are transported in symport with the substrate.

Uptake of L-glutamate via $GltS_{Ec}$ was studied in membrane vesicles of *E. coli* ECOMUT1/pMK15 ($GltS_{Ec}$). Glutamate uptake was only observed when Na^+ was present in the assay buffer, irrespective of pH. Under these conditions and at pH 6.0, L-glutamate uptake was partially

Table 2. Inhibition constants of glutamate transport[a] in membrane vesicles of *E. coli* ECOMUT1 harbouring pECO118 ($GltP_{Ec}$), pMK15 ($GltS_{Ec}$), pGBT112 ($GltT_{Bs}$) or pGBT231 ($GltT_{Bc}$).

	K_i^{app} (μM)			
Inhibitor	$GltP_{Ec}$	$GltS_{Ec}$	$GltT_{Bs}$	$GltT_{Bc}$
L-Glutamate	8	37	21	83
L-Aspartate	7	>1000	8	110
L-Glutamine	>1000	>1000	>1000	>1000
L-Asparagine	>1000	>1000	>1000	>1000
D-Glutamate	650	450	650	900
αMG[b]	>1000	950	>1000	>1000
βHA[b]	20	>1000	59	210
Cysteate	13	>1000	108	187

a. Uptake experiments were performed by diluting membrane vesicles 100-fold into oxygen-saturated 50 mM potassium phosphate, pH 6.0, 5 mM $MgSO_4$, 50 mM NaCl, 10 mM K-asc and 100 μM PMS. After 1 min of incubation, L-[^{14}C]-glutamate (1.9 μM) plus a 500-fold excess of inhibitor was added and transport assays were further handled as described in the *Experimental procedures.* (Stock solutions of the inhibitors were prepared in 50 mM potassium phosphate pH 6.0, 5 mM $MgSO_4$ and 50 mM NaCl.)
b. αMG, α-methyl-glutamic acid; βHA, β-hydroxy aspartic acid.

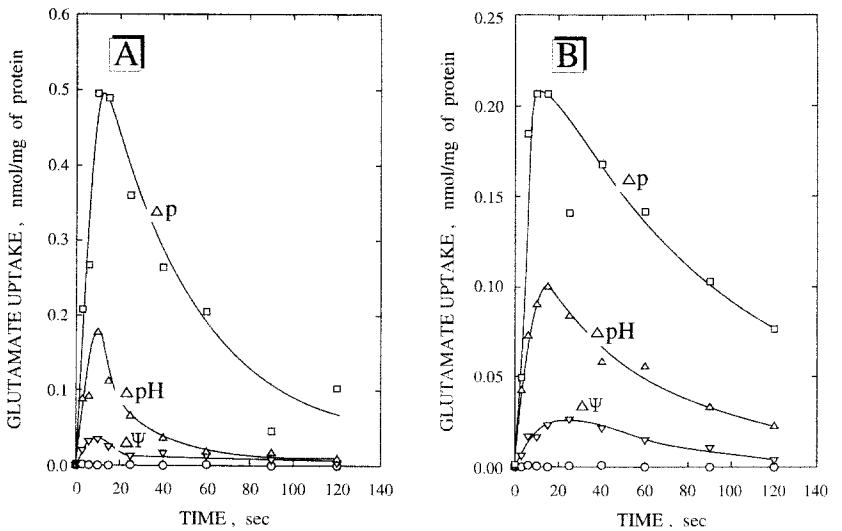

Fig. 2. Uptake of L-glutamate in membrane vesicles of ECOMUT1/pGBT118 ($GltP_{Ec}$) (A) and ECOMUT1/pGBT112 ($GltT_{Bs}$) (B) driven by artificially imposed ion gradients. L-Glutamate uptake was measured in the presence of a Δp (□), ΔpH (△) or ΔΨ (▽) as described in the *Experimental procedures*. The assay temperature was 37°C. Control experiments were performed by diluting the membrane vesicles 100-fold into the buffer in which the membranes were resuspended (○).

inhibited by monensin (electroneutral Na^+/H^+ exchange) and nigericin, whereas valinomycin had no effect on the rate of transport. Complete inhibition of L-glutamate uptake was observed in the presence of nigericin plus valinomycin. Under these conditions, not only the Δp but also the ΔpNa is abolished. At pH 8.0 when the proton motive force is largely composed of an electrical potential, monensin as well as nigericin did not affect L-glutamate transport, whereas valinomycin abolished transport completely (data not shown). These findings suggest that transport of L-glutamate mediated by $GltS_{Ec}$ is an electrogenic process in which only Na^+ is co-transported.

The cation-coupling mechanism of the GltT transporters, expressed in *E. coli*, differs from previous observations in *B. stearothermophilus* and *B. caldotenax* (De Vrij *et al.*, 1989; Heyne *et al.*, 1991). The isolation of glutamate transport mutants of *B. caldotenax*, using βHA as a toxic analogue, yielded transport phenotypes that were consistent with the inactivation of a single gene product, i.e. GltT (data not shown). To address the apparent discrepancy between the cation-coupling mechanisms of GltT in *E. coli* (this study) and previous studies, L-glutamate transport was analysed further in membrane vesicles of *B. stearothermophilus* and *B. caldotenax*. The effects of ionophores in the *Bacillus* membranes were indeed different from those observed in *E. coli* membrane vesicles. In the *Bacillus* vesicles and at pH 6.0, nigericin did not affect transport, whereas dissipation of the ΔΨ (membrane potential) by valinomycin inhibited L-glutamate transport partially. Furthermore, an approximately threefold increase in the initial rate of L-glutamate transport was observed in the presence of monensin, possibly as a result of the conversion of the ΔpH into a ΔpNa. These results suggest that L-glutamate transport in *B. stearothermophilus* and *B. caldotenax* is electrogenic and are consistent with the previously reported Na^+–H^+–L-glutamate co-transport (De Vrij *et al.*, 1989; Heyne *et al.*, 1991).

Artificial ion gradients

Since L-glutamate is an anionic species at physiological pH, the electrogenic nature of the transport processes in membrane vesicles of ECOMUT1 harbouring pECO118 ($GltP_{Ec}$), pGBT112 ($GltT_{Bs}$), pGBT231 ($GltT_{Bc}$) or pMK15 ($GltS_{Ec}$), as well as in membrane vesicles of *B. stearothermophilus* and *B. caldotenax*, suggests that at least two cations are transported in symport with the substrate. Although the use of ionophores allows the selective manipulation of the components of the proton and sodium motive force across the membrane, dissipation of one of the components usually results in a partially compensatory increase of another component. Furthermore, the ionophores may affect transport not only through their effects on the driving force but also by altering parameters such as the internal pH (Poolman *et al.*, 1987). To specify the nature of the co-transported cation(s) more precisely, experiments were carried out in which L-glutamate uptake was driven by artificially imposed ion gradients.

The Δp as well as its components ΔΨ and ΔpH, were able to drive L-glutamate uptake in membrane vesicles of ECOMUT1 harbouring pECO118 ($GltP_{Ec}$), pGBT112 ($GltT_{Bs}$) or pGBT231 ($GltT_{Bc}$) (Fig. 2). An Na^+ gradient, either alone or in addition to an artificially generated Δp, ΔΨ or ΔpH, had no effect on L-glutamate uptake. The results at 45°C were similar to those obtained at 37°C (data not shown). Again, these data strongly suggest that $GltP_{Ec}$, $GltT_{Bs}$ and $GltT_{Bc}$ only transport protons in symport with L-glutamate. L-Glutamate transport by ECOMUT1 harbouring pMK15 ($GltS_{Ec}$) was only observed when a ΔpNa was generated; transport was not observed when only Δp,

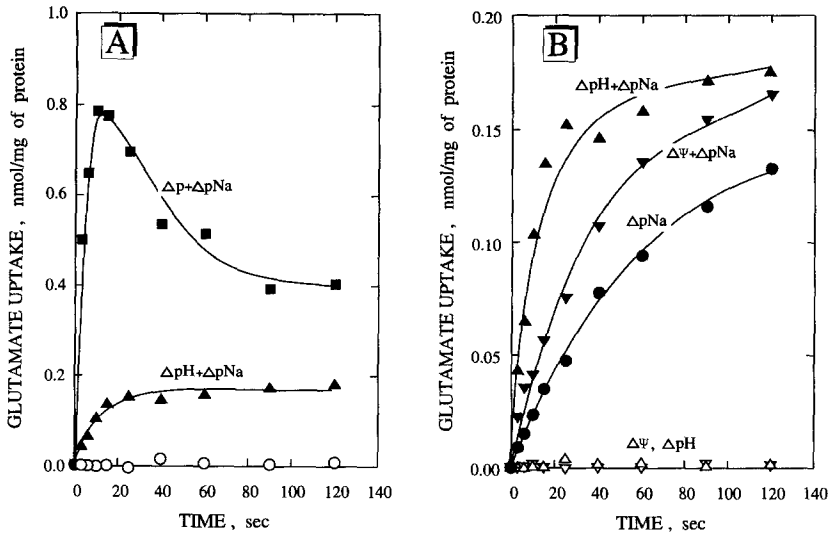

Fig. 3. Uptake of L-glutamate in membrane vesicles of ECOMUT1/pMK15 ($GltS_{Ec}$) driven by artificially imposed ion gradients. L-Glutamate uptake was measured in the presence of a Δp + ΔpNa (■) or ΔpH + ΔpNa (▲) (A); and a ΔpNa (●), ΔΨ + ΔpNa (▼), ΔpH + ΔpNa (▲), ΔpH (△) or ΔΨ (▽) (B), as described in the *Experimental procedures*. The assay temperature was 37°C. Control experiments were performed by diluting the membrane vesicles 100-fold into the buffer in which the membranes were resuspended (○).

ΔΨ or ΔpH was imposed (Fig. 3B). The generation of a Δp, ΔΨ or ΔpH on top of a ΔpNa stimulated the transport (Fig. 3). These results are in accordance with the notion that Na^+ is obligatory for L-glutamate uptake mediated by $GltS_{Ec}$. The stimulatory effect of ΔpH on ΔpNa driven L-glutamate transport may reflect an activation of $GltS_{Ec}$ by an increased internal pH (Poolman *et al.*, 1987).

The Δp as well as its components ΔΨ and ΔpH were able to drive L-glutamate uptake in membrane vesicles of *B. stearothermophilus* and *B. caldotenax*. Furthermore, uptake of L-glutamate was enhanced several-fold when a ΔpNa was applied in addition to Δp, ΔΨ or ΔpH (Fig. 4). Since *B. stearothermophilus* and *B. caldotenax* are thermophilic organisms, the effect of temperature on the ΔpNa stimulation of L-glutamate transport was studied. Interestingly, the results revealed that the stimulation of transport by a ΔpNa was highly dependent on temperature (Fig. 5). The data demonstrate that, in addition to H^+, Na^+ also plays a role in GltT-mediated L-glutamate uptake in membrane vesicles of *B. stearothermophilus* and *B. caldotenax*.

Discussion

In this study, we report the construction of an *E. coli* strain (ECOMUT1) which is devoid of secondary glutamate transport activity. The GltP and GltS proteins of *E. coli* and GltT of *B. stearothermophilus* and *B. caldotenax* have been expressed in this strain and their functional properties with regard to substrate specificity, cation selectivity and nature of the driving force for transport have been investigated. An important conclusion that follows from these studies is that the cation specificity and the apparent affinity constant for substrate uptake is highly

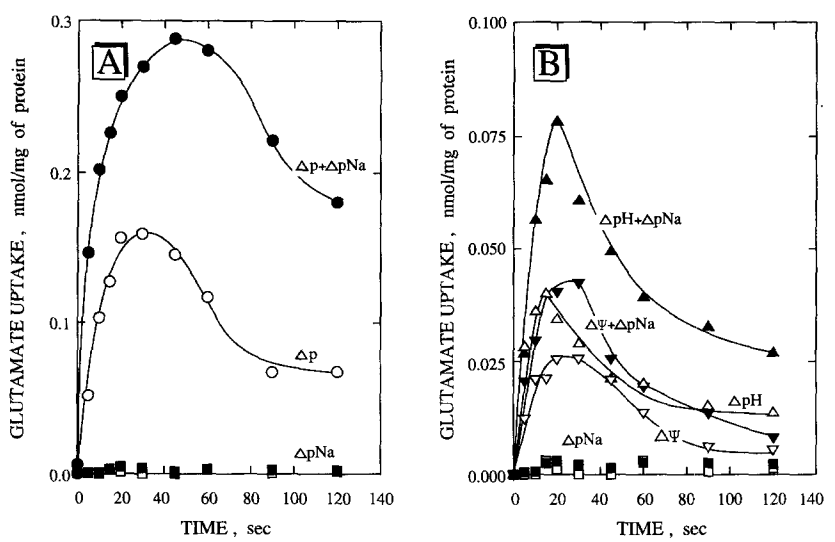

Fig. 4. Uptake of L-glutamate in membrane vesicles of *B. stearothermophilus* ($GltT_{Bs}$) driven by artificially imposed ion gradients. L-Glutamate uptake was measured in the presence of a ΔpNa (■), Δp (○) or Δp + ΔpNa (●) (A); and a ΔpNa (■), ΔpH (△), ΔpH + ΔpNa (▲), ΔΨ (▽) or ΔΨ + ΔpNa (▼) (B), as described in the *Experimental procedures*. The assay temperature was 45°C. Control experiments were performed by diluting the membrane vesicles 100-fold into the buffer in which the membranes were resuspended (□).

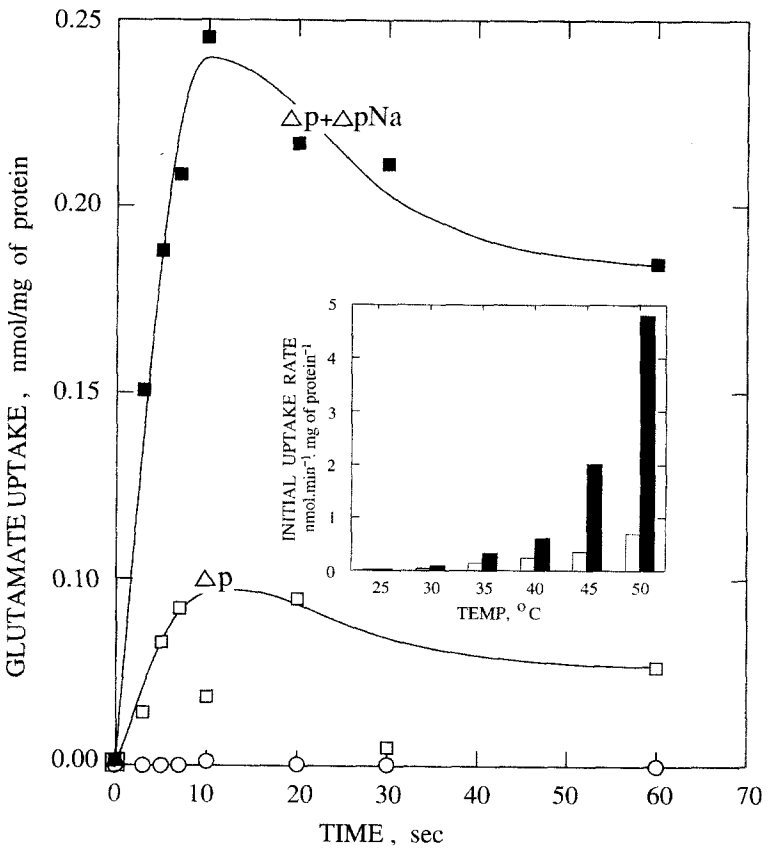

Fig. 5. Temperature-dependent Na^+ stimulation of L-glutamate uptake driven by artificially imposed ion gradients in membrane vesicles of *B. caldotenax* ($GltT_{Bc}$). Uptake of L-glutamate was measured at 45°C in the presence of a Δp (□) or Δp + ΔpNa (■) as described in the *Experimental procedures*. Control experiments were performed by diluting the membrane vesicles 100-fold into the buffer in which the membranes were resuspended (○). The effect of temperature on the initial uptake rate in the presence (black bars) and absence (open bars) of ΔpNa is shown (inset).

dependent on the lipid environment in which the proteins are expressed.

Studies in membrane vesicles of *E. coli* ECOMUT1 harbouring pECO118 ($GltP_{Ec}$), pGBT112 ($GltT_{Bs}$) or pGBT231 ($GltT_{Bc}$) and of *B. stearothermophilus* and *B. caldotenax* revealed that the GltP- and GltT-type L-glutamate transporters are specific for the substrates L-glutamate and L-aspartate (Table 2). The K_m^{app} values for L-glutamate and L-aspartate are in the micromolar range (Table 1). For $GltT_{Bs}$ and $GltT_{Bc}$ the K_m^{app} values are approximately 10-fold higher when the proteins are expressed in *E. coli* as compared with the *Bacillus* strains. L-Glutamate transport via $GltP_{Ec}$, $GltT_{Bs}$ and $GltT_{Bc}$ is inhibited by β-HA and cysteic acid, as previously reported for $GltP_{Ec}$ of *E. coli* (Schellenberg and Furlong, 1977). In contrast to Deguchi *et al.* (1989), who characterized $GltP_{Ec}$ of *E. coli* B, no significant inhibition of L-glutamate uptake by L-glutamine and L-asparagine was found (at a 500-fold excess over L-[^{14}C]-glutamate). Since only the sequence of *gltP* of *E. coli* K-12 is known (Tolner *et al.*, 1992b), it cannot be excluded that differences in the amino acid sequences of GltP from *E. coli* strain K-12 and B are responsible for the observed differences. The inhibitor of GltS α-MG (Schellenberg and Furlong, 1977) did not affect transport of the $GltP_{Ec}$, $GltT_{Bs}$ and $GltT_{Bc}$. The substrate specificity of these L-glutamate transporters is similar to that of GltP in *B. subtilis* (Tolner *et al.*, 1995).

Studies in membrane vesicles of ECOMUT1 harbouring pMK15 ($GltS_{Ec}$) revealed that the GltS of *E. coli* K-12 is highly specific for L-glutamate (Table 2). Our data indicate a low affinity of GltS for D-glutamate (K_i^{app} 450 μM), whereas L-glutamine does not significantly inhibit L-glutamate uptake. These observations are at variance with those of Deguchi *et al.* (1989) in *E. coli* B, who reported a strong inhibition of GltS-mediated L-glutamate transport by D-glutamate and L-glutamine. The *gltS*-coding regions of *E. coli* K-12 and B differ in six bases, but these differences are translationally silent (Deguchi *et al.*, 1990; Kalman *et al.*, 1991) and therefore cannot be responsible for the discrepancies in substrate specificity. Surprisingly, GltS exhibited a low affinity for α-MG, which is often reported to be a specific inhibitor of GltS-mediated transport in *E. coli* (Deguchi *et al.*, 1990; 1989; Kalman *et al.*, 1991; Schellenberg and Furlong, 1977).

L-Glutamate transport in membrane vesicles of *B. stearothermophilus* ($GltT_{Bs}$), *B. caldotenax* ($GltT_{Bc}$) and *E. coli* ECOMUT1 harbouring pECO118 ($GltP_{Ec}$), pMK15 ($GltS_{Ec}$), pGBT112 ($GltT_{Bs}$) or pGBT231 ($GltT_{Bc}$) was found to be electrogenic and to occur in symport with at least two cations. This was shown by the effect of ionophores on

K-asc/PMS (or *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD))-energized transport as well as by transport driven by artificially imposed ion gradients (Figs 2–4). The studies have revealed distinct differences with respect to the nature of coupling ion(s) to transport. (i) $GltS_{Ec}$ transports L-glutamate in symport with at least two Na^+. The involvement of Na^+ in GltS-mediated transport has been suggested before (Frank and Hopkins, 1969; Kahane *et al.*, 1975; Marcus and Halpern, 1969; Miner and Frank, 1974; Schellenberg and Furlong, 1977), but in most of these studies a role of protons as alternative coupling ion (Hasan and Tsuchiya, 1977; MacDonald *et al.*, 1977; Tsuchiya *et al.*, 1977) or a symport of L-glutamate with Na^+ plus H^+ could not be excluded (Fujimura *et al.*, 1983a,b). By complementation of the *E. coli* ECOMUT1 strain ($GltS^-$, $GltP^-$) and the use of membrane vesicles, we have shown that only $GltS_{Ec}$ is responsible for the observed Na^+ dependency of L-glutamate transport in *E. coli*. (ii) A surprising observation was that $GltT_{Bs}$ and $GltT_{Bc}$, when expressed in *E. coli*, transport L-glutamate in symport with at least two H^+. Previous studies on the energetics of these transporters in their native environment revealed a H^+–Na^+ coupled symport mechanism (De Vrij *et al.*, 1989). The observations made for $GltP_{Ec}$ mimic those of the GltP protein of *B. subtilis*, which has recently been identified as an electrogenic H^+–glutamate symporter (Tolner *et al.*, 1995). (iii) When $GltT_{Bs}$ and $GltT_{Bc}$ are expressed homologously in the corresponding *Bacillus* strains, a ΔpNa indeed stimulates L-glutamate transport, i.e. when a $\Delta\Psi$, ΔpH or Δp are generated simultaneously. Furthermore, the stimulation of the initial rate of L-glutamate uptake by a ΔpNa increased from threefold at 25°C to sevenfold at 55°C. Under the conditions in which a ΔpNa was generated, no increase in the $\Delta\Psi$ and/or ΔpH was observed (data not shown) which could have occurred as a result of Na^+/H^+ antiport activity. If Na^+ is taken up by a Na^+/H^+ antiporter, the ΔpH (inside alkaline) can be raised as a result of the coupled efflux of H^+ in the antiport reaction. Since this was not observed, the effect of Na^+ must be exerted on the GltT proteins directly.

It is concluded that, in the *E. coli* lipid environment, L-glutamate is probably transported with two H^+ while in the *Bacillus* lipid environment L-glutamate transport is mediated with one H^+ and one Na^+. Apparently, the conformation of the GltT transporters in *E. coli* is such that H^+ (H_3O^+; Boyer, 1988), but not Na^+, can be accommodated in the cation-binding pocket. These observations need further clarification at the molecular level. For such analysis, membrane vesicles are too complex and studies of the purified and reconstituted L-glutamate transporters are needed.

Finally, the present study calls for precautions when the cation selectivity of transporters is studied in membranes (heterologous expression or reconstituted into artificial membranes) that are different from the native environment.

Experimental procedures

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used are listed in Table 3. *B. stearothermophilus* was grown at 63°C, with vigorous aeration in a medium containing 2% (w/v) tryptone, 1% (w/v) yeast extract and 170 mM NaCl, and adjusted to pH 7.0. *E. coli* strains were grown at 37°C with vigorous aeration in Luria–Bertani (LB) or M9 media (Sambrook *et al.*, 1989). The mineral media were supplemented with essential nutrients as indicated by the auxotrophic markers. When needed, carbenicillin, kanamycin, chloramphenicol, X-gal and IPTG were added to a final concentration of 100, 20 and 30 $\mu g\,ml^{-1}$, 20 $mg\,ml^{-1}$ and 100 μM, respectively.

Isolation of membrane vesicles

For transport studies in membrane vesicles, cells of *B. stearothermophilus* or *B. caldotenax* were grown to an A_{660} of 1.0. Cells were harvested and membrane vesicles were isolated essentially as described by Konings *et al.* (1973), except that the incubation temperature of the cells in the presence of lysozyme was increased to 50°C. Cells of *E. coli* strain ECOMUT1 harbouring plasmid pKK223-3, pECO118, pGBT112 or pMK15 were grown to an A_{660} of 1.0 in M9 (containing 1% (v/v) LB and 1 mM IPTG), and membrane vesicles were isolated as described previously by Kaback (1971). Cytoplasmic membranes of *B. stearothermophilus*, *B. caldotenax* and *E. coli* were resuspended to about 15 mg protein per ml in 50 mM potassium phosphate pH 6.0 and stored in liquid nitrogen.

Transport assays: Na^+ and H^+ motive force-driven uptake in E. coli *membrane vesicles*

Uptake of L-[^{14}C]-Glutamate was assayed at 37°C, under continuous aeration. The electron donor system K-asc/PMS was used to generate Δp. Membrane vesicles were diluted 100-fold into 50 mM potassium phosphate pH 6.0, 5 mM $MgSO_4$, 10 mM K-asc and 100 μM PMS (< 30 μM Na^+). The effect of the sodium gradient (ΔpNa) was assessed by adding 50 mM NaCl to this assay buffer. When appropriate, valinomycin (2 μM), nigericin (1 μM) or monensin (20 nM) was added to abolish the ion gradients across the membrane. After 1 min of incubation, the uptake was initiated by adding L-[^{14}C]-glutamate to a final concentration of 1.9 μM. The uptake reactions were terminated by adding a 10-fold excess of ice-cold 0.1 M KCl, followed by immediate filtration over cellulose nitrate filters (0.45 μm pore size). The filters were washed once with 2 ml ice-cold KCl.

Transport assays: Na^+ and H^+ motive force-driven uptake in vesicles from Bacillus *sp.*

The assays were similar to those described above, except that K-asc (10 mM)/TMPD (100 μM) was used as electron donor system; the temperature was kept at 50°C.

Table 3. Bacterial strains, plasmids and phages used.

Bacterial Strain/ Plasmid	Relevant characteristics	Source/Reference
Strain		
B. stearothermophilus		ATCC 7954
E. coli		
JM101	Δ(*lac–proAB*)(F′*lacI*^{q} ΔM15)	Yanisch-Perron *et al.* (1985)
EC101	JM101, RepA$^+$, Km^R	Department of Genetics, Groningen University
MK416	GltS$^-$, Km^R	Kalman *et al.* (1991)
ECOMUT1	MK416 :: pORI557CM (inserted in *gltP*), GltS$^-$, GltP$^-$, Km^R, Cm^R, Tc^R	This work
CJ236	*dut*$^-$, *ung*$^-$, Cm^R	Laboratory collection
Plasmid		
pUC18/19	Ap^R	Yanisch-Perron *et al.* (1995)
pSKII$^-$	Ap^R	Stratagene
pTAQI	LacI$^+$, Ap^R (expression vector)	Laboratory collection
pORI24	RepA$^-$, only replicates in RepA$^+$ strains, Tc^R	Department of Genetics, Groningen University
pHP45ΩCMR	Carrying a Cm^R gene on a 3.7 kb fragment in the multiple cloning site, Ap^R, Cm^R	Fellay *et al.* (1987)
pMK15	pSKII, carrying $gltS_{EcK\text{-}12}$, Ap^R	Kalman *et al.* (1991)
pBW8	pT7-6, carrying $gltP_{EcK\text{-}12}$, Ap^R	Wallace *et al.* (1990)
pGBT102	pUC18, carrying $gltT_{Bs}$, Ap^R	Tolner *et al.* (1992a)
pGBT112	pUC18, carrying $gltT_{Bs}$, (in reverse orientation relative to pGBT102), Ap^R	Tolner *et al.* (1992a)
pGBT231	pUC18, carrying $gltT_{Bc}$, Ap^R	Tolner *et al.* (1992a)
pGBT521	pUC18, carrying $gltP_{EcK\text{-}12}$, Ap^R	This work
pGBT521S	pUC18, carrying $gltP_{EcK\text{-}12}$, *Sma*I at position 479, Ap^R	This work
pBST111	pSKII$^-$ (*Eco*RV digested), carrying *gltT* promoter/RBS region of pBST100 (PCR product: T7 universal and M13 reverse primer), Ap^R	This work
pECO103	pUC19 (*Sma*I/*Bam*HI digested), carrying *gltP* promoter/RBS region of pECO100, Ap^R	This work
pECO113	pECO103 (*Ava*I/*Eco*RI digested), carrying *gltP* terminator region of pGBT521, Ap^R	This work
pECO116	pECO113 (*Xba*I/*Sac*I digested), carrying *gltT* promoter/RBS region of pBST100, Ap^R	This work
pECO118	pTAQI (*Hin*dIII/*Sac*I digested), carrying *gltT*/*gltP* fragment of pECO116, Ap^R	This work
pGBT550	pUC19 (*Ava*I/*Hin*cII digested), carrying a 926 bp *Ava*I–*Hin*cII *gltP* fragment of pGBT521, Ap^R	This work
pGBT557	pSKII$^-$ (*Kpn*I/*Hin*cII digested), carrying the *Kpn*I–*Hin*cII *gltP* fragment of pGBT550, Ap^R	This work
pGBT557CM	pGBT557 (*Eco*RI digested), carrying the Cm^R gene (*Eco*RI fragment) of pLGL19, Ap^R, Cm^R	This work
pORI557CM	pORI24, carrying the *Kpn*I–*Pst*I fragment of pGBT557CM (*gltP* fragment and Cm^R gene), Ap^R, Cm^R, Tc^R	This work
Phages		
M13mp18/19		Yanisch-Perron *et al.* (1985)
pBST102	M13mp19, carrying the *Eco*RI–*Hin*cII *gltT* fragment of pGBT102	This work
pBST100	pBST102, *Xba*I immediately upstream of the start codon	This work
pECO521	M13mp18, carrying the *Bam*HI–*Bam*HI *gltP* fragment of pGBT521	This work
pECO100	pECO521, *Xba*I immediately upstream of the start codon	This work

Ap^R, Cm^R and Tc^R, ampicillin, chloramphenicol and tetracycline resistant, respectively.

Transport assays: artificial ion gradients

Transport of L-glutamate driven by artificial gradients was assayed essentially as described previously (Tolner *et al.*, 1995). Membrane vesicles were washed twice in 20 mM morpholine-ethanesulphonic acid (Mes), 100 mM acetic acid (HAc) plus 100 mM KOH (adjusted to pH 6.0 with H_2SO_4) and subsequently incubated for 2 h at 4°C in the same buffer. After centrifugation for 5 min at 200 000 × *g*, the membranes were concentrated to approximately 40 mg protein per ml. Uptake driven by specific ion gradients was initiated by diluting the membrane vesicles 100-fold into the appropriate buffer containing L-[^{14}C]-glutamate (1.9 μM) with or without valinomycin (2 nmol mg^{-1}). In addition, the buffers contained for Δp, 120 mM Mes, 100 mM methylglucamine (Mglu) (+ valinomycin); ΔΨ, 20 mM Mes, 100 mM HAc, 100 mM Mglu (+ valinomycin); ΔpH, 120 mM Mes, 100 mM KOH (+ valinomycin); ΔpNa, 20 mM Mes, 100 mM HAc, 100 mM NaOH; ΔpNa + ΔΨ, 20 mM Mes, 100 mM HAc, 100 mM NaOH (+ valinomycin); ΔpNa + ΔpH, 120 mM Mes, 100 mM NaOH (+ valinomycin); and ΔpNa + Δp, 120 mM Mes, 100 mM NaOH (+ valinomycin). The buffers were adjusted to pH 6.0 with Mglu or H_2SO_4 and all contained 5 mM $MgSO_4$. Control experiments were performed by diluting the membrane vesicles 100-fold into the buffer in which the membranes were resuspended (MES/HAc/KOH). The reaction was terminated

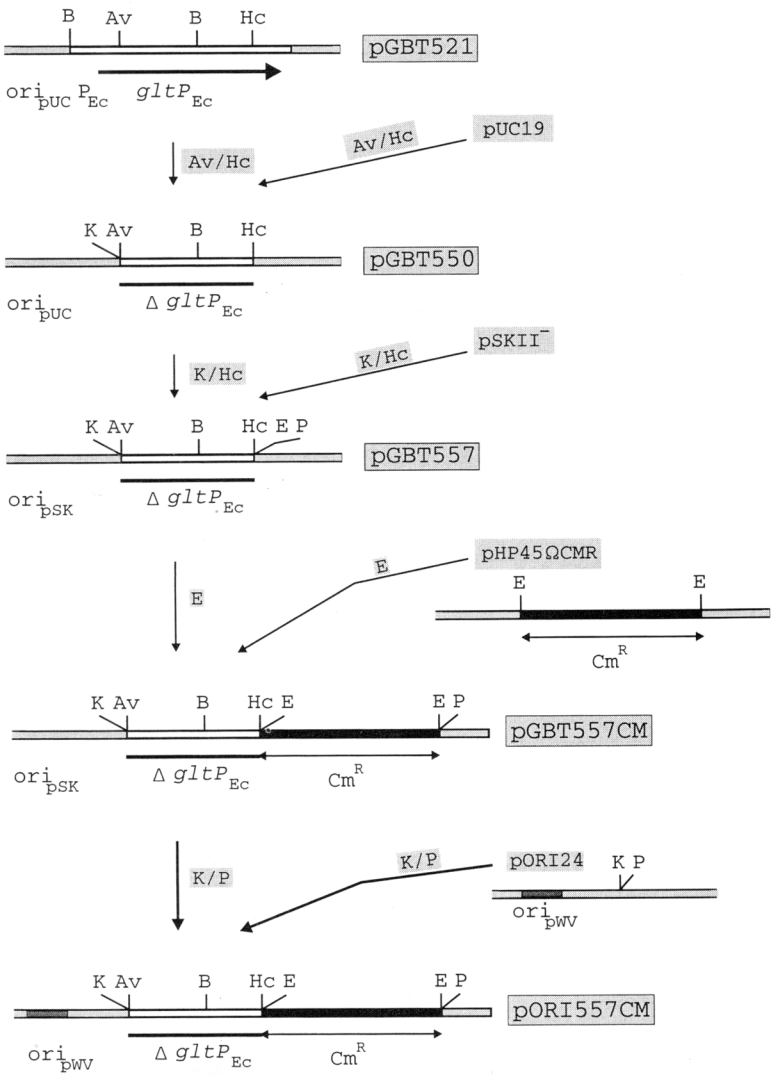

Fig. 6. Construction of plasmid pORI557CM. The internal 926 bp *AvaI–HincII gltP* fragment of pGBT521 was ligated into *AvaI–HincII*-digested pUC19. Subsequently, the resulting plasmid (pGBT550) was *KpnI–HincII*-digested and the *gltP*-containing fragment was ligated into the compatible sites of pSKII$^-$ to form pGBT557. After ligation of the Cm^R gene containing the *EcoRI* fragment of pHP45ΩCMR into *EcoRI*-linearized pGBT557, the resulting plasmid, pGBT557CM, was digested with *KpnI* and *PstI*. The *KpnI–PstI* fragment of pGBT557CM was ligated into *KpnI–PstI* linearized pORI24 to yield pORI557CM.
Symbols as in Fig. 1. In addition, K represents *KpnI* and P *PstI*. Black, open and shaded box: Cm^R gene, *gltP* (plus promoter/RBS) and vector sequence, respectively. Arrow below sequence: coding region of respective gene. Ori$_{pUC}$, ori$_{pSK}$ and ori$_{pWV}$ represent origin of replication of pUC19, pSKII$^-$ and pWV01, respectively.

as described above. Care was taken to avoid contamination of the buffers with Na^+; disposable plastic materials and ultrapure chemicals were used in all experiments (concentration of Na^+ contamination < 10 μM). The uptake experiments were performed at 37°C or 50°C for *E. coli* and *B. stearothermophilus* (and *B. caldotenax*), respectively, unless stated otherwise.

The kinetic parameters of transport, apparent K_m and V_{max}, were estimated from the uptake of (labelled) amino acid (2 to 400 μM) determined after 10 s. Results were analysed by fitting the data to the Michaelis equation.

DNA manipulations

Mini- and large-scale preparations of plasmid DNA were obtained by the alkaline lysis method (Birnboim and Doly, 1979; Ish-Horowicz and Burke, 1981). Chromosomal DNA was isolated essentially as decribed previously (Leenhouts *et al.*, 1990), except that mutanolysine was omitted. The strains were transformed by heat-shock after rubidium chloride treatment of the cells (Sambrook *et al.*, 1989) or by electrotransformation (Dower *et al.*, 1988). Site-directed mutagenesis was performed according to Kunkel (1985). Other DNA manipulations were performed as described (Sambrook *et al.*, 1989). Polymerase chain reactions (PCR) (Mullis and Falona, 1987) were carried out with Vent DNA polymerase (New England BioLabs), using the recommended buffer, 20 ng primers, 200 ng phage DNA (M13 replicative form) and 400 μM deoxynucleoside triphosphates in a total volume of 100 μl and 35 cycles of 1 min at 94°C, 1 min at 50°C and 1 min at 70°C.

Construction of strain ECOMUT1

An *E. coli gltS*$^-$, *gltP*$^-$ strain was constructed by Campbell-like integration of vector pORI557CM into the chromosome of *E. coli* MK416 (*gltS*$^-$); pORI557CM carries an internal fragment of the *E. coli gltP* gene (Fig. 6, for details). *E. coli* MK416 was transformed with pORI557CM and transformants were selected on LB agar plates containing 20 μg ml^{-1} kanamycin and 30 μg ml^{-1} chloramphenicol. The disruption of the *gltP* gene was confirmed by Southern blot analysis (using an

*Aval–Hin*cII internal *gltP* fragment and *Pst*I-linearized pORI24 as probes).

Sequence determination

The nucleotide sequence of both strands of PCR products and of fragments that had been subject to site-directed mutagenesis was determined by the dideoxy-chain termination method (Sanger *et al.*, 1977). Double-stranded DNA was sequenced using a T7 sequencing kit (Pharmacia). PCGene (release 6.26, Genofit) was used for computer-assisted sequence analysis. The amplified and cloned fragments were in all cases identical to the sequences reported in the original papers, except for the specific mutations that were introduced (see legends to Figs 1 and 6 for details).

Protein determination

Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

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